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(54) Title: PROCESS FOR THE INTRODUCTION OF EXOGENOUS DNA IN SOMATIC AND GERM ANIMAL CELLS

(57) Abstract

A process is described for the introduction of exogenous DNA into somatic and germ animal cells: the DNA, exogenous or modified according to known techniques of recombinant DNA, is introduced into the animal spermatozoa which are to be modified and said spermatozoa are employed for egg fertilization according to usual artificial fertilization techniques.

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PROCESS FOR THE INTRODUCTION OF EXOGENOUS DNA IN SOMATIC AND GERM ANIMAL CELLS

1. Field of the invention

The present invention refers to a process for the introduction of exogenous DNA into somatic and germ animal cells.

In particular, the process consists in introducing DNA, exogenous or modified according to known techniques of recombinant DNA, into the spermatozoa of the animal which is to be modified and in employing said spermatozoa for egg fertilization according to known techniques for artificial fertilization.

2.Prior technique

The creation of transgenic animals, that is of animals in which are permanently integrated genetic informations extraneous to their own genomes and deriving from other genetic systems, has been and still is an objective of primary importance for the study of genetic regulation, both for chemical and therapeutical ends and for breeding domestic mammals, fish, echinoderma and amphibia.

It is possible in fact to create animals with particular advantageous characteristics, such as e.g. rate of growth or resistance to certain diseases in the case of animals for breeding, or, viceversa, predisposition to certain diseases in the case of animals utilized for experimenting new drugs. The first attempts at obtaining transgenic animals go back to the middle of the seventies. Those attempts were chiefly based on the manipulation of mice embryos or of cultured cells and on the direct DNA (e.g. SV 40)

introduction into the mice blastocyst cavities, as described by M.H.L. & A. Mc Laren in Experimental Cell Research (1924) 86, 1-8, and by R. Jaenisch & B. Mintz (1974) in Proc. Natl. Acad. Sci. USA (1974) 71, 1250-54.

The results obtained were, however, disappointing,particularly because of the difficulty of the employed techniques.

In 1980 John W. Gordon et al. described in Proc. Natl. Acad. Sci USA (1980), 77 No. 12, 7380-84 a new technique for the genetic transformation of mice embryos by microinjection of cloned DNA into the pronucleus of fertilized oocytes.

The thus treated zygotes were re-implanted in the uterus of a pseudogravid mother and the gestation brought to term.

By means of this technique, which was refined in the last years, it was shown that it is possible to introduce exogenous DNA into mouse somatic and germ cells obtaining transgenic animals.

A typical example of transgenic animals obtained by the technique of the microinjection of exogenous DNA into fertilized eggs, and employed for experimenting anti-tumoral therapies, are the mice described in the U.S.P. 4.736.866, which show in their somatic and germ cells the presence of an oncogenic sequence.

The microinjection technique is being experimented in the field of breeding; howevwer, we do not have information of finally acquired results.

The DNA microinjection in fertilized oocytes is at any rate a complex and expensive technique, with low efficiency because of the

high rate of abortions, the high rate of mosaicism in the obtained animals and the marked sterility of the same.

3. The technical problem

The fundamental technical problem which is solved by the present invention is the introduction of DNA,treated according to known techniques of recombinant DNA,into the cellules of an animal pertaining to an animal species which does not actually posses the typical sequences of the introduced exogenous DNA, with the result that the genetic informations contained in said recombinant DNA is permanently integrated in the genomes of the treated individual and may therefore be transmitted to the successive progeny of the individual.

D. Detailed description of the invention

The process for the introduction of cloned DNA into the cells of a different species according to the present invention is based on an experimental observation, namely the surprising easiness with which molecules, even if of large dimensions, succeed in penetrating into the spermatozoa head.

This property, typical of spermatozoa both of mammals and of other animal species, was utilized to modify the spermatozoa, intorducing in them the cloned DNA to be transferred.

With the modified spermatozoa, the corresponding oocytes are then fertilized by means of the artificial fertilization techniques employed with unmodified spermatozoa.

According to a fundamental characteristic of the present invention,

the spermatozoa of the species into which one wants to introduce the genetic informations extraneous to its own genomes, and originating from other systems, are utilized as vectors for transferring the extraneous DNA.

One of the possible ways of performing the process according to the present invention comprises the following steps:

a) preparation of an aqueous spermatozoa suspension

b) transformation of the spermatozoa with cloned DNA

c) "in vitro" fertilization of the oocytes by means of the modified spermatozoa

d) implantation of the fertilized oocytes into pseudogravid females of the selected species.

According to another possible way of performing the present invention, the modified spermatozoa may be employed directly for the animal fertilization without going through the "in vitro" oocyte fertilization and successive implantation of the same into pseudogravid females.

This form of realization of the invention is particularly useful in case the process is employed for breeding.

The process according to the invention is much simpler, much more rapid and less costly than the microinjection technique, and does not bring about abortions which are very frequent in the microinjection technique.

The invention will now be described in detail with particular reference to the modification of mice spermatozoa.

We have employed this animal because all the laboratory technique for its "in vitro" fertilization and for the study of the integration and expression of its genes are amply reported in the scientific literature.

As exogenous DNA we used p SV2 CAT, Polyoma and the human growth gene, because their restriction maps are described in literature and comprise base sequences which are not naturally present in mouse genome.

The identification of these sequences in the "positive" mouse, that is in the mouse obtained from the egg fertilized with the treated spermatozoa, allows to ascertain without the shadow of a doubt that the cloned DNA was actually introduced into the treated spermatozoa and through these into the fertilized eggs and therefore integrated into the genome of the resulting transgenic individuals.

a) preparations of the spermatozoa.

A spermatozoa suspension was prepared by pressing the epididymis of a male mouse into 1 ml PM buffer (prepared as described by D.G. Whittingam. Culture of Mouse - ove - (1971) - J Reprod. Fert. Supp. 14, p.7-21).

The spermatozoa suspension was centrifuged so to separate the spermatozoa which were again suspendend in 1 ml of buffer.

The above treatment was repeated 5 times so to "wash" the spermatozoa by assuring the complete elimination of seminal liquor.

The buffer was modified eliminating sodium lactate, penicillin and steptomycin, substituting monosodiumphosphate by 0.15 mM

bisodiumphosphate and changing the amount of sodium chloride to 120 mM. The spermatozoa suspension was then diluted to a concentration of 1-2 million/ml and incubated for a period of from 30 minutes to 3 hrs at a temperature of from 20° to 37°C, in air containing more than 5% to 10% of carbon dioxide.

b) DNA transformation of the spermatozoa

To the diluted spermatozoa suspension, in fractions of 500 µl, is added the solution of the cloned, circular DNA which is intended to be inserted into the spermatozoa, and the mixture is further incubated for at least 30 minutes at a temperature of from 0° to 37°C, in air containing more than 5% to 10% of carbon dioxide.

At the lower temperatures, the insertion of DNA into the spermatozoa is easier, due to a reduced activity of endogenous nuclease of spermatozoa, but the incubation time is longer than 30 minutes.

The cloned circular DNA solution is added in an amount such as to obtain a final concentration into the cloned DNA mixture comprised between 0.4 and 2 µg/ml.

c) egg fertilization

Mature mouse females are induced to superovulate by means of human choronic gonadotropin and the eggs are extracetd from the oviduct 14.5 hours after the injection (according to the method described by B. Hogan et al. in "Manipulating the Mouse Embryo A Laboratory Manual" CSM New York, 1986).

The eggs extracted from the oviducts are introduced into the 500 µl fractions of the transformed spermatozoa and incubated from 5 to 10

hours at a temperature of from 20° to 37°C, in air containing more than 5% to 10% of carbon dioxide. At the end of said period, the eggs are washed with M16 buffer (prepared as described by Whittingam -see point a) supra) and left for an entire night in 50 µl of the same buffer.

After 24 hours the embryos are surgically transferred, at the stage of two cellules, into the oviducts of pseudogravid females.

The offsprings deriving from these implants, at the age of three weeks, are amputated of a terminal tail fragment, from which the DNA is extracted which is analyzed with the aid of the "Southern blot" described in the book "Molecular Cloning": A Laboratory Manual" by T. Maniatis et al. - C.S.M., New York 1984.

This analysis allows to identify "positive" individuals, that is those whose genome posses, integrated or in episomic form, one or more copies of the same cloned DNA introduced into the starting spermatozoa.

The yield of "positive"individuals obtained following the process of the present invention is always higher than 30% up to 70%, and, what is more, no sterile individuals are found among them.

The successive genetic characterization of the positive animals is carried out with the two analysis methods of restriction and sequence.

The analysis of the genome DNA of positive mice was carried out according to two methods:

Restriction Analysis

DNA restriction analysis by means of specific restriction enzymes which allow the subdivision of genome DNA into fragments. The obtained fragments are then fractionated by electrophoresis and transferred onto a nitrocellulose filter according to the known Southern blot technique. The filter is then treated with the probe specific for the initial cloned DNA employed for transforming the spermatozoa, made radioactive with P^{32} and exposed on a x-ray sensitive film.

The film will be exposed in correspondence with the sites where the radioactive probe was bound to the filter, that is in correspondence with each radioactive DNA bond, leaving a signal for each DNA fragment.

The presence of one or more signals, their number and the dimensions of the DNA fragments which they represent allow to conclude that sequences exist which are analogous to the positive mouse genome probe, and to determine a restriction map.

The analogy between this map and the one of the cloned DNA introduced into the spermatozoa from which the "positive" mouse originates proves that the original clone sequences are integrated into the transgenic mouse genome.

- Sequence analysis

To analize the sequence of the bases constituing the "positive" mouse genome, equal amounts (15 µg) of genome DNA of a positive mouse and of the pUC13 plasmid resistant to ampicillin were restricted with the restriction EcoRI enzyme.

The two restricted DNA were then mixed, recircularized and introduced into Eschericia Coli HB101 bacteria, which were then cultivated on Agar + Ampicillin.

The positive colonies (that is the ones containing a cloned fragment) were separated, amplified and purified. The cloned fragment was then separated from the pUC13 vector by restriction with EcoRI and 626 bases were sequened from it using the Langer method.

It was thus possible to ascertain that the initial clone was transferred from the spermatozoa into the fertilized egg and then integrated into the genoma of the resulting individual.

Beside the two methods reported above, we have carried out an anlysis of the spermatozoa after their transformation with the cloned DNA, to the end of ascertaining the location of the exogenous DNA.

To this end we employed H^3 labeled DNA, and various aliquots of the spermatozoa solutions after their incubation with labeled DNA were radio-autographed at the optical and at the electronic microscope.

The obtained results have evidenced that cloned DNA is specifically located inside the spermatozoa head in sub-equatorial position.

Traces of radioactivity in other regions of the spermatozoa are insignificant.

As it is known that the acrosomal fusion reaction between spermatozoa and oocyte at the moment of fertilization (with transferral of genetic material from spermatozoa to the egg) takes

place in that particular region, it is believed that also the exogenous DNA is trasferred at the same time and in the same way as the DNA proper of the species. This mechanism might explain the surprising efficiency of the process according to the invention.

CLAIMS

1. Process for the introduction of exogenous DNA into somatic and germ animal cells characterized by the introduction of said DNA, exogenous or modified according to known DNA recombinant techniques, into the spermatozoa of the animal which one intends to modify, and by the use of said spermatozoa for fertilizing eggs according to usual artificial fertilization techniques.

2. Process according to claim 1, characterized in that:

a) a water spermatozoa suspension is prepared;

b) the spermatozoa are modified with the cloned DNA;

c) oocytes are fertilized "in vitro" by means of the modified spermatozoa;

d) the fertilized oocytes are implanted into pseudogravid females of the selected species.

3. Process according to claim 2, characterized in that said aqueous spermatozoa suspension is buffered with a FM buffer, diluted to a spermatozoa concentration of 1-2 millions/ml and incubated at 20° to 37°C for a period of from 30 minutes to 3 hours, in air containing more than 5% up to 10% of carbon dioxide.

4. Process according to claim 2, characterized in that the circular cloned DNA solution to be inserted into the spermatozoa is added to said incubated aqeuous spermatoza suspension and incubated further for at least 30 minutes at a temperature of from 0° to 37°C, said solution being added in an amount such as to have a final cloned DNA concentration in the mixture of from 0.4 to 2 µg/ml.

5. Process according to claim 2, characterized in that the oocytes to be fertilized are added to the aqueous incubated spermatozoa suspension and the mixture is incubated for 5 to 10 hours at a temperature of from 20° to 37°C, in air containing more than 5% to 10% of carbon dioxide.

6. Process according to claim 5, characterized in that the embrious obtained from the fertilized oocytes, as described, when reaching the development stage of two cells are surgically transferred into the oviducts of pseudogravid females.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 90/00032

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 15/87

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	WO, A, 87/05325 (TRANSGENE GmbH) 11 September 1987 see pages 29-33 --	1,2,5,6
X	Proceedings of the National Academy of Sciences, volume 68, no. 2, February 1971, (US), B.G. Brackett et al.: "Uptake of heterologous genome by mammalian spermatozoa and its transfer to ova through fertilization", pages 353-357 see the whole article --	1
P,X	Cell, volume 57, 2 June 1989, Cell Press, M. Lavitrano et al.: "Sperm cells as vectors for introducing foreign DNA into Eggs: genetic transformation of mice", pages 717-723 see the whole article --	1-6

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* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21st March 1990	24.04.90
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EUROPEAN PATENT OFFICE	F.W. HECK

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
T	Cell, volume 59, 20 October 1989, Cell Press, R.L. Brinster et al.: "No simple solution for making transgenic mice", pages 239-241 see the whole letter and reply	1-6

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9000032 SA 33170

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 11/04/90. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8705325	11-09-87	DE-A- 3636991	24-09-87
		AU-A- 7167987	28-09-87
		EP-A- 0258427	09-03-88
		JP-T- 63502638	06-10-88
		ZA-A- 8701468	17-08-87